REMARKS

Favorable reconsideration of this application as presently amended is respectfully requested.

Claims 1-3, 5, 7-12, 15-19, and 26-27 stand rejected under 35 U.S.C. 102(b) as being anticipated by Saros *et al.* (U.S. Patent No. 4,853,336) for reasons of record. This rejection is respectfully traversed with respect to the claims as amended.

Claim 1 as currently presented claims the feature of a flow cytometry apparatus that includes a flow cytometer for selectively analyzing particles in each of a plurality of samples as a fluid flow stream passes through the flow cytometer. Although the September 13, 2001, Office Action states that "the flow analyzer taught by Saros appears to disclose the same components as the flow cytometer recited in claim 1" (see Office Action, p. 4), in fact, Saros *et al.* neither teaches nor suggests the use of a flow cytometer for analyzing particles, as claimed in claim 1.

Although Saros *et al.* may describe the detection of analytes in a liquid sample in a conventional detection system, in the detection device described in Saros *et al.*, the fluids pass through these detection devices in a single bulk phase so that alignment of particles is not an issue and air bubbles are inconsequential. In contrast, in a flow cytometer, the detection device of the present invention, there are 2 fluid phases: a narrow hydrodynamically focused core sample stream that carries the sample particles and an outer sheath stream of much larger volume. Large air bubbles may cause sustained disturbance of the hydrodynamic focusing resulting in sustained misalignment of the sample stream, *i.e.* the sample stream no longer passes precisely through the laser beam; and therefore, a sustained mismeasurement of particle fluorescence may occur. In order to ensure a steady stream of aligned cells, hydrodynamic focusing is required (see Hoy, "An Introduction to Flow Cytometry" (copy attached)). Because of the issues with respect to hydrodynamic focusing, it has generally been thought that keeping air out of a fluid flow stream containing particles is extremely important, because the presence of air has been viewed disrupting the alignment of the particles in the fluid flow stream, thereby making the particles

"unmeasurable" by the flow cytometer. In contrast, detection devices other than flow cytometers do not require such focusing and, therefore, have been understood to allow for the presence of air bubbles in a fluid flow stream being analyzed.

Although it may have been known to use air bubbles to separate samples when using detection systems that do not employ a flow cytometer, such as Saros *et al.*, prior to Applicants' invention as claimed in claim 1, it was absolutely not obvious to use an air bubble as a sample separator with a flow cytometer. In fact, in the flow cytometer art it was considered desirable to remove any air bubbles before the air bubbles go through a flow cytometer. However, Applicants have discovered that by controlling the size of air bubbles used to separate samples, the disruption of hydrodynamic focusing can be prevented thereby allowing a flow cytometer to be used with a fluid flow stream containing air bubbles, as claimed by claim 1.

Also, prior to Applicants' invention, as claimed in claim 1, flow cytometers have only been used to analyze one sample at time, because putting buffer fluids between samples did not prevent intermixing and because of the above-described problems with using bubbles to separate samples.

For the reasons discussed above, claim 1 is patentable over Saros *et al.*

Claims 2-3, 5, 7, 9-12, 15-19, and 26-27 depend directly or indirectly from claim 1, and, accordingly, include all of the patentable features of claim 1 as well as other patentable features. Therefore, claims 2-3, 5, 7, 9-12, 15-19, and 26-27 are patentable over Saros *et al.* for at least the reasons discussed above with respect to claim 1.

With respect to claim 8, the above rejection has been rendered moot by the cancellation of claim 8.

With respect to new claims 46 and 47, claims 46 and 47 each claim a flow cytometry apparatus in which a portion of a fluid flow stream containing samples separated by a separation gas is contained within a tube having an internal diameter of 0.02 inches or less when the fluid flow stream passes through a peristaltic pump.

Saros *et al.* neither teaches nor suggests the unexpected advantages to be obtained by using a tube having an internal diameter of 0.02 inches or less in a flow cytometry apparatus. However, as noted in Larry Sklar's June 20, 2001, Declaration Under 37 C.F.R. § 1.132 (submitted with Applicants' June 21, 2001 Amendment), experiments conducted in his laboratory indicated "that integrity of bubbles used to separate samples is important for proper sample separation in the flow cytometry apparatus of the present invention" (See Sklar Declaration, paragraph 6) and "that the integrity of the bubbles used to separate samples in the flow cytometer of the present invention is compromised when the inner diameter of the tubing is greater than 0.02 inches" (See Sklar Declaration, paragraph 7). As has been held by the Court of Appeals for the Federal Circuit, "[o]ne way for a patent applicant to rebut a prima facie case of obviousness is to make a showing of 'unexpected results,' *i.e.*, to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected." *In re Soni*, 34 USPQ2d 1684, 1687 (Fed. Cir. 1995).

Applicants have unexpectedly discovered that tubing having an inner diameter of 0.02 inches or less has the unexpected property of preserving the integrity of the bubbles, separation gas, used to separate samples in the flow cytometry apparatus of claim 46 or claim 47. Therefore, Applicants have shown that the claimed range of 0.02 inches or less achieves unexpected results relative to the teachings of the prior art. In fact, Saros *et al.* provides no teaching or suggestion with respect to controlling the inner diameter of a tube used in a flow cytometry apparatus, much less teaching or suggesting the advantages of claim 46's or claim 47's range. Also, contrary to what is stated in the Office Action, Applicants have not merely discovered an "optimum of workable ranges" (See Office Action, p. 8), but have in fact discovered that the claimed range provides substantially improved results, the standard set forth by the Federal Circuit in *In re Soni*, 34 USPQ2d 1684,1688 (Fed. Cir. 1995). Therefore, claims 46 and 47 are patentable over Saros *et al.*

Based on paragraph 4 of the September 13, 2001, Office Action, claims 4, 6, 13-14, and 20-24 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Saros *et al.* in view of Kercso *et al.* (U.S. Patent No. 6,132,685) for the reasons of record (the March 28, 2001, Office Action). This rejection is respectfully traversed.

Claims 4, 6, 13-14, 20-24 and new claims 46 and 47 are dependent on claim 1, either directly or indirectly, and, therefore, include claim 1's patentable features of using a flow cytometer for selectively analyzing particles in each of a plurality of samples, separated from each other by a separation gas, as the fluid flow stream passes through the flow cytometer. Kercso *et al.* is only cited for showing the use of "multiwell microtiter plates" and microfluidic channels "fabricated on [a] planar substrate comprising polymeric materials which are inherently hydrophobic such as polyvinylchloride (PVC) and polyurethane" as set forth in the March 28, 2001, Office Action at pp. 6-7. (The March 28, 2001, Office Action provides the "reasons of record" referred to in the rejection set forth at paragraph 4 of the September 13, 2001, Office Action). Therefore, Kercso *et al.* cannot remedy the deficiency of Saros *et al.* with respect to failing to describe or show the use of a flow cytometer used for analyzing particles in each of a plurality of samples in a fluid flow stream. Therefore, claims 4, 6, 13-14, 20-24 and new claims 46 and 47 are patentable over the combination of Kercso *et al.* with Saros *et al.*

Additionally, with respect to new claims 46 and 47, as discussed above, Saros *et al.* fails to teach or suggest claim 46's and claim 47's feature of a tube having an inner diameter of 0.02 inches or less. Kercso *et al.* is only cited for showing the use of "multiwell microtiter plates" and microfluidic channels "fabricated on [a] planar substrate comprising polymeric materials which are inherently hydrophobic such as polyvinylchloride (PVC) and polyurethane" as set forth in the March 28, 2001, Office Action at pp. 6-7. Also, as noted in the March 28, 2001, Office Action, Saros *et al.* and Kercso *et al.* fail to disclose "an inner diameter of 0.02 inches (See March 28, 2001, Office Action, p. 7). Therefore, Kercso *et al.* does not remedy the deficiencies of Saros *et al.* with respect to failing to teach an inner diameter of 0.02 inches or less and thus claim 1 is patentable over the combination of Kercso *et al.* with Saros *et al.* and claims 46 and 47 are patentable over the combination of Kercso *et al.* with Saros *et al.*

In addition, with respect to claims 1-27, these claims have been rejected on the basis of facts within the personal knowledge of the Examiner. The Examiner has asserted that "the flow analyzer for selectively analyzing a plurality of samples taught

by Saros appears to disclose the same components as the flow cytometer recited in claim 1" (see Office Action, p. 4). However the Examiner has not indicated to Applicants which specific features of Saros *et al.* correspond to the components of a flow cytometer, so the Applicants do not know which components of Saros the Examiner believes correspond to the flow cytometer of claim 1. Accordingly, under 37 C.F.R. § 1.104(d)(2), Applicants hereby request that the Examiner provide an affidavit supporting the Examiner's assertion used as a basis for the rejections based on Saros *et al.*

Based on paragraph 8 of the September 13, 2001, Office Action, claim 25 stands rejected under 35 U.S.C. 103(a) as being unpatentable over Saros *et al.* in view of Kercso *et al.* and in further view of Farrell *et al.* (U.S. Patent No. 5,788,927) for the reasons of record (the March 28, 2001, Office Action). This rejection is respectfully traversed.

Claim 25 is indirectly dependent on claim 1 and therefore includes claim 1's patentable features of using a flow cytometer for selectively analyzing particles in each of a plurality of samples, separated from each other by a separation gas, as the fluid flow stream passes through the flow cytometer. Farrell *et al.* is only cited for suggesting an inverted mounting design of a well plate (See March 28, 2001, Office Action, p. 11). Therefore, Farrell *et al.* does not remedy the deficiencies of the combination of Kercso *et al.* and Saros *et al.* with respect to failing to describe or show the use of a flow cytometer used for analyzing particles in each of a plurality of samples in a fluid flow stream. Therefore, claim 25 is patentable over the combination of Farrell *et al.* with Kercso *et al.* and Saros *et al.*

Based on paragraph 9 of the September 13, 2001, Office Action, claims 1-3, 8-12, 5-19, and 26-27 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Parce *et al.* (U.S. Patent No. 6,150,180) in view of Hach *et al.* (U.S. Patent No. 4,053,282) or Trinel *et al.* (U.S. Patent No. 4,116,631) for the reasons of record (the March 28, 2001, Office Action). This rejection is respectfully traversed with respect to the claims as amended.

In the Office Action the following is stated with respect to justifying combining Hach *et al.* or Trinel *et al.* with Parce *et al.*:

> One of ordinary skill in the art at the time of the instant invention would have a reasonable expectation of success in substituting air to separate individual samples for analysis in flow analyzers or microfluidic systems such as taught by Hach or Trinel for the spacer buffer or separation fluid in the flow channels taught by Parce because Hach and Trinel specifically suggested that separation gas, when incorporated into proper tubing materials an parameter requirements, provides adequate separation between sequential samples so as to prevent contamination or carry-over therebetween. (See September 13, 2001, Office Action, p. 7)

The above-quoted statements used as a basis for combining Hach *et al.* or Trinel *et al.* with Parce *et al.* indicate that the combination of Hach *et al.* or Trinel *et al.* with Parce *et al.* is *prima facie* improper. The above statements cite no portion of Parce that would supply a person of ordinary skill in the art with a motivation to combine the teachings of Hach *et al.* or Trinel *et al.* with the teachings of Parce. As stated by the Federal Circuit in *Sibia Neurosciences Inc. v. Cadus Pharmaceutical Corp.* 55 USPQ2d 1927, 1931 (Fed. Cir. 2000), "the factual underpinnings of obviousness include whether a reference provides a motivation to combine its teachings with another," citing *Tec Air, Inc. v. Denso Mfg.*, 52 USPQ2d 1296, 1298 (Fed. Cir. 1999). Accordingly, the teachings of Hach *et al.* and Trinel *et al.* may not be properly combined with the teachings of Parce *et al.*

Also, the above-quoted statements fail to provide a motivation for combining the teachings of Parce *et al.* with Hach *et al.* or Trinel *et al.* The fact that Hach *et al.* and Trinel *et al.* may suggest that separation gas may provide adequate separation between sequential samples provides no motivation for the person of ordinary skill in the art to combine Parce *et al.*'s high throughput screening assay with Hach *et al.*'s method and apparatus for sampling impure water or Trinel *et al.*'s method for microbiological analysis of liquid mediums.

Based on the above-quoted statements, the rejection of claims 1-3, 8-12, 5-19, and 26-27 based on Parce *et al.* in view of Hach *et al.* or Trinel *et al.* improperly relies on Applicants' specification and hindsight to provide the "motivation" to combine these cited references. But for reading Applicants' specification, a person of ordinary skill in the art who read Parce *et al.* would have no motivation to look at Hach *et al.* or Trinel *et al.* nor would the person of ordinary skill who read Hach *et al.* or Trinel *et al.* be motivated to look at Parce *et al.* As held in *In re Dembiczak*, "[c]ombining prior art references without evidence of such a suggestion, teaching, or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability—the essence of hindsight. *See, e.g., Interconnect Planning Corp. v. Feil,* 774 F.2d 1132, 1138, 227 USPQ 543, 547 (Fed. Cir. 1985) ("the invention must be viewed not with the blueprint drawn by the inventor, but in the state of art that existed at that time")." Furthermore, as held in *Sibia Neurosciences v. Cadus Pharmaceutical Corp.*, "[c]are must be taken to avoid hindsight reconstruction by using 'the patent in suit as a guide through the maze of prior art references, combining the right reference in the right way so as to achieve the result of the claims in suit'", citing *Grain Processing Corp. v. American Maize-Product Co.*, 5 USPQ2d 1788, 1792 (Fed. Cir. 1988), *Sibia Neurosciences v. Cadus Pharmaceutical Corp.,* 55 USPQ2d 1927, 1934 (Fed. Cir. 2000).

Based on the above-cited cases, there is no proper motivation to combine the teachings of Hach *et al.* or Trinel *et al.* with Parce *et al.* and the rejection of claims 1-3, 8-12, 5-19, and 26-27 rejecting claim 1 under 35 USC § 103(a) based on a combination of Hach *et al.* or Trinel *et al.* with Parce *et al.* is improper and should be withdrawn.

Also, claim 1 as currently presented claims the feature of a flow cytometry apparatus that includes a flow cytometer for selectively analyzing particles in each of a plurality of samples as a fluid flow stream passes through the flow cytometer. Although the September 13, 2001, Office Action states that "the flow analyzer taught by Parce appears to disclose the same components as the flow cytometer recited in claim 1" (see Office Action, p. 4), in fact, Parce *et al.* neither teaches nor suggests the use of a flow cytometer for analyzing particles, as claimed in claim 1.

Although Parce *et al.* may describe a method of screening test compounds using a conventional detecting system, in the detection device described in Parce *et al.*, the fluids pass through the detection device in a single phase so that alignment of particles is not an issue and air bubbles are inconsequential. In contrast, in a flow cytometer, the detection device of the present invention, there are 2 fluid phases: a narrow hydrodynamically focused core sample stream that carries the sample particles and an outer sheath stream of much larger volume. Large air bubbles may cause sustained disturbance of the hydrodynamic focusing resulting in sustained misalignment of the sample stream, *i.e.* the sample stream no longer passes precisely through the laser beam; and therefore, a sustained mismeasurement of particle fluorescence may occur. In order to ensure a steady stream of aligned cells, hydrodynamic focusing is required (see Hoy, "An Introduction to Flow Cytometry" (copy attached)). Because of the issues with respect to hydrodynamic focusing, it has generally been thought that keeping air out of a fluid flow stream containing particles is extremely important, because the presence of air has been viewed disrupting the alignment of the particles in the fluid flow stream, thereby making the particles "unmeasurable" by the flow cytometer. In contrast, detection devices other than flow cytometers do not require such focusing and, therefore, have been understood to allow for the presence of air bubbles in a fluid flow stream being analyzed.

Although it may have been known to use air bubbles to separate samples when using detection systems that do not employ a flow cytometer, such as Parce *et al.*, prior to Applicants' invention as claimed in claim 1, it was absolutely not obvious to use an air bubble as a sample separator with a flow cytometer. In fact, in the flow cytometer art, it was considered desirable to remove any air bubbles before the air bubbles went through a flow cytometer. However, Applicants have discovered that by controlling the size of air bubbles used to separate samples, the disruption of hydrodynamic focusing can be prevented thereby allowing a flow cytometer to be used with a fluid flow stream containing air bubbles, as claimed by claim 1.

Also, prior to Applicants' invention as claimed in claim 1, flow cytometers have only been used to analyze one sample at a time, because putting buffer fluids between samples did not prevent intermixing and because of the above-described problems with using bubbles to separate samples.

For the reasons discussed above, claim 1 is patentable over Parce *et al.*

Hach *et al.* is only cited for disclosing "a pump and a means for periodically injecting a separation gas (air bubbles) in the sample tubing to separate liquids and clean sweep the tubing" as set forth in the March 28, 2001, Office Action at p. 11. (The March 28, 2001, Office Action provides the "reasons of record" referred to in the rejection set forth at paragraph 9 of the September 13, 2001, Office Action). Therefore, Hach *et al.* does not remedy the deficiencies of Parce *et al.* with respect to failing to describe or show claim 1's feature of using a flow cytometer as a detection device and claim 1 is thus patentable over the combination of Hach *et al.* with Parce *et al.*

Trinel *et al.* is only cited for disclosing "an automatic flow analysis apparatus wherein samples are separated by intermediate segments of decontamination solution and wherein spacing between the samples and the decontamination solution are effected by segments of an separation inert gas [sic]" as set forth in the March 28, 2001, Office Action at p. 11. (The March 28, 2001, Office Action provides the "reasons of record" referred to in the rejection set forth at paragraph 9 of the September 13, 2001, Office Action). Therefore, Trinel *et al.* does not remedy the deficiencies of Parce *et al.* with respect to failing to describe or show claim 1's feature of using a flow cytometer as a detection device and thus claim 1 is patentable over the combination of Trinel *et al.* with Parce *et al.*

Claims 2-3, 9-12, 15-19, 26-27 and new claims 46 and 47 depend directly or indirectly from claim 1, and, accordingly, include all of the patentable features of claim 1 as well as other patentable features. Therefore, claims 2-3, 9-12, 15-19, 26-27 and new claims 46 and 47 are patentable over the combination of Hach *et al.* or Trinel *et al.* with Parce *et al.* for the reasons discussed above with respect to claim 1.

With respect to claim 8, the above rejection has been rendered moot by the cancellation of claim 8.

Additionally, with respect to new claims 46 and 47, each of claims 46 and 47 claims a flow cytometry apparatus in which a portion of a fluid flow stream containing samples separated by a separation gas is contained within a tube having an internal diameter of 0.02 inches or less when the fluid flow stream passes through a peristaltic pump. However, Parce *et al.* neither teaches nor suggests the unexpected advantages to be obtained by using a tube having an internal diameter of 0.02 inches or less in a flow cytometry apparatus. As noted in Larry Sklar's June 20, 2001, Declaration Under 37 C.F.R. § 1.132 (submitted with Applicants' June 21, 2001, Amendment), experiments conducted in his laboratory indicated "that integrity of bubbles used to separate samples is important for proper sample separation in the flow cytometry apparatus of the present invention" (See Sklar Declaration, paragraph 6) and "that the integrity of the bubbles used to separate samples in the flow cytometer of the present invention is compromised when the inner diameter of the tubing is greater than 0.02 inches" (See Sklar Declaration, paragraph 7). As has been held by the Court of Appeals for the Federal Circuit, "[o]ne way for a patent applicant to rebut a prima facie case of obviousness is to make a showing of 'unexpected results,' *i.e.*, to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected." *In re Soni*, 34 USPQ2d 1684, 1687 (Fed. Cir. 1995).

Applicants have unexpectedly discovered that tubing having an inner diameter of 0.02 inches or less has the unexpected property of preserving the integrity of the bubbles, separation gas, used to separate samples in the flow cytometry apparatus of claims 46 and 47. Therefore, Applicants have shown that the claimed range of 0.02 inches or less achieves unexpected results relative to the teachings of the prior art. In fact, Parce *et al.* provides no teaching or suggestion with respect to controlling the inner diameter of a tube used in a flow cytometry apparatus, much less teaching or suggesting the advantages of claim 46's or 47's range. In fact, because Parce *et al.* fails "to disclose a means for introducing a separation gas between each of the plurality of samples", as admitted in the March 28, 2001, Office Action (see March 28, 2001, Office Action, p. 11), Parce *et al.* can teach nothing about using particular inner tube diameters to improve bubble integrity between samples. Also, contrary to what is stated in the Office Action, Applicants have not merely discovered an "optimum of workable ranges" (See September 13, 2001, Office Action, p. 8), but

have in fact discovered that the claimed range provides substantially improved results, the standard set forth by the Federal Circuit in *In re Soni*, 34 USPQ2d 1684,1688 (Fed. Cir. 1995). Therefore, claims 46 and 47 are patentable over Parce *et al.*

Hach *et al.* is only cited for disclosing "a pump and a means for periodically injecting a separation gas (air bubbles) in the sample tubing to separate liquids and clean sweep the tubing" as set forth in the March 28, 2001, Office Action at p. 11. (The March 28, 2001, Office Action provides the "reasons of record" referred to in the rejection set forth at paragraph 9 of the September 13, 2001, Office Action). Therefore, Hach *et al.* does not remedy the deficiencies of Parce *et al.* with respect to failing to teach an inner diameter of 0.02 inches or less and claims 46 and 47 are thus patentable over the combination of Hach *et al.* with Parce *et al.*

Trinel *et al.* is only cited for disclosing "an automatic flow analysis apparatus wherein samples are separated by intermediate segments of decontamination solution and wherein spacing between the samples and the decontamination solution are effected by segments of an separation inert gas [sic]" as set forth in the March 28, 2001, Office Action at p. 11. (The March 28, 2001, Office Action provides the "reasons of record" referred to in the rejection set forth at paragraph 9 of the September 13, 2001, Office Action). Therefore, Trinel *et al.* does not remedy the deficiencies of Parce *et al.* with respect to failing to teach an inner diameter of 0.02 inches or less and claims 46 and 47 are thus patentable over the combination of Trinel *et al.* with Parce *et al.*

In addition, with respect to claims 1-27, these claims have been rejected on the basis of facts within the personal knowledge of the Examiner. The Examiner has asserted that "the flow analyzer for selectively analyzing a plurality of samples as suggested by the combined disclosures of Parce in view of Hach or Trinell [sic] appears to suggest the same components as the flow cytometer recited in claim 1 which therefore qualifies their combined function as being able to perform cytometric measurements" (see Office Action, p. 4). However, the Examiner has not indicated to Applicants which specific features of Parce *et al.*, Hach *et al.* and Trinel *et al.* correspond to the components of a flow cytometer, so the Applicants do not know which components of Parce *et al.*, Hach *et al.* and Trinel *et al.* the Examiner believes

correspond to the flow cytometer of claim 1. Accordingly, under 37 C.F.R. § 1.104(d)(2), Applicants hereby request that the Examiner provide an affidavit supporting the Examiner's assertion used as a basis for the rejections based on Parce *et al.*, Hach *et al.* and Trinel *et al.*

If the Examiner has any questions or concerns regarding the present response, the Examiner is invited to contact Mark J. Guttag at 703-591-2664. In view of the foregoing, it is respectfully submitted that this application is now in condition for allowance, and favorable action is respectfully solicited.

Respectfully submitted,

Mark J. Guttag
Reg. No. 33,057

enc.: HOY, Terry, "An Introduction of Flow Cytometry."

May 13, 2002

JAGTIANI + GUTTAG
Democracy Square Business Center
10379-B Democracy Lane
Fairfax, Virginia 22030
(703) 591-2664

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	)
SKLAR *et al.*	)
	) **Art Unit: 1641**
Serial No.: 09/501,643	)
	) **Examiner: Gabel, G.**
Filed: February 10, 2000	)
	)
For: FLOW CYTOMETRY FOR HIGH THROUGHPUT SCREENING	) **DOCKET NO: UNME-0070-1**

Director of the U.S. Patent and Trademark Office
Washington, D.C. 20231

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Sir:

Below are the amendments in the accompanying Amendment for the above-identified application shown in redlined format:

IN THE CLAIMS:

Please amend the claims, without prejudice or disclaimer, as shown below:

Please cancel claim 8 without prejudice or disclaimer.

1. (Twice Amended) A flow cytometry apparatus for the detection of particles from a plurality of samples comprising:

means for moving the plurality of samples comprising particles from a plurality of respective source wells into a fluid flow stream, said means for moving the plurality of samples comprising a pump;

means for introducing a separation gas between each of said plurality of samples in said fluid flow stream; and

a flow cytometer~~means~~ for selectively analyzing said particles in each of said plurality of samples ~~for said particles~~as said fluid flow stream passes through said flow cytometer ~~in a flow cytometer~~.

2. (Amended) The flow cytometry apparatus of claim 1, wherein said means for moving said plurality of samples further comprises an autosampler.

3. The flow cytometry apparatus of claim 2, wherein said autosampler includes a probe and said flow cytometry apparatus includes a means for exposing a probe tip of said probe to a jet of gas to remove liquid from said probe tip.

4. The flow cytometry apparatus of claim 2, wherein said autosampler includes a probe having a conical tip.

5. The flow cytometry apparatus of claim 2, wherein said autosampler includes a hydrophobic probe.

6. The flow cytometry apparatus of claim 5, wherein said probe comprises a hydrophobic material.

7. The flow cytometry apparatus of claim 5, wherein said probe is coated with a hydrophobic material.

9. (Amended) The flow cytometry apparatus of claim ~~8~~1, ~~wherein a portion of said fluid flow stream passing through said peristaltic pump is contained within~~ wherein said tube comprises a high speed multi-sample tube.

10. (Amended) The flow cytometry apparatus of claim ~~8~~1, wherein said pump comprises a peristaltic pump ~~is located along said fluid flow stream between said autosampler and said means for selectively analyzing said plurality of samples~~.

11. (Amended) The flow cytometry apparatus of claim 10, further comprising a single length of tubing extending from said autosampler to said ~~means for selectively analyzing said plurality of samples~~flow cytometer.

12. (Amended) The flow cytometry apparatus of claim 11, wherein said single length of tubing comprises a high speed multi-sample ~~tubing~~tube.

13. (Twice Amended) The flow cytometry apparatus of claim ~~12~~1, wherein said high speed multi-sample ~~tubing~~ tube comprises a poly vinyl chloride tube~~tubing having an inner diameter about 0.01 to about 0.03 inches and a wall thickness of about 0.01 to about 0.03 inches~~.

14. (Twice Amended) The flow cytometry apparatus of claim ~~12~~1, wherein said high speed multi-sample ~~tubing~~ tube comprises a poly vinyl chloride ~~tubing~~ tube having an inner diameter about 0.02 inches and a wall thickness of about 0.02 inches.

15. The flow cytometry apparatus of claim 1, wherein said separation gas comprises air.

16. The flow cytometry apparatus of claim 1, wherein said plurality of samples are homogenous.

17. The flow cytometry apparatus of claim 1, wherein said plurality of samples are heterogeneous.

18. The flow cytometry apparatus of claim 1, wherein said particles comprise biomaterials.

19. The flow cytometry apparatus of claim 18, wherein said biomaterials are fluorescently tagged.

20. The flow cytometry apparatus of claim 1, further comprising a well plate including said plurality of respective source wells.

21. The flow cytometry apparatus of claim 20, wherein said well plate includes at least 96 source wells.

22. The flow cytometry apparatus of claim 20, wherein said well plate includes at least 384 source wells.

23. The flow cytometry apparatus of claim 20, wherein said well plate includes at least 1536 source wells.

24. The flow cytometry apparatus of claim 20, wherein said well plate includes wells having a conical shape.

25. The flow cytometry apparatus of claim 20, wherein said well plate is mounted in an inverted position.

26. The flow cytometry apparatus of claim 1, further comprising a means for injecting a buffer fluid between adjacent samples in said fluid flow stream.

27. The flow cytometry apparatus of claim 1, wherein at least one of said plurality of samples includes a drug present therein.

Please add the following new claims :

46. (New) The flow cytometry apparatus of claim 1, wherein a portion of said fluid flow stream passing through said pump is contained within a tube having an internal diameter of 0.02 inches or less.

47. (New) The flow cytometry apparatus of claim 10, wherein a portion of said fluid flow stream passing through said peristaltic pump is contained within a tube having an internal diameter of 0.02 inches or less.

AN INTRODUCTION TO FLOW CYTOMETRY

Terry Hoy, Haematology Department, UWCM, Cardiff, UK
Telephone 029 2074 3458 FAX 029 2074 4655
E-mail hoy@cf.ac.uk

Last revision: 18 August 2000 15:44

PRINCIPLES OF FLOW CYTOMETRY

DATA INTERPRETATION AND ANALYSIS

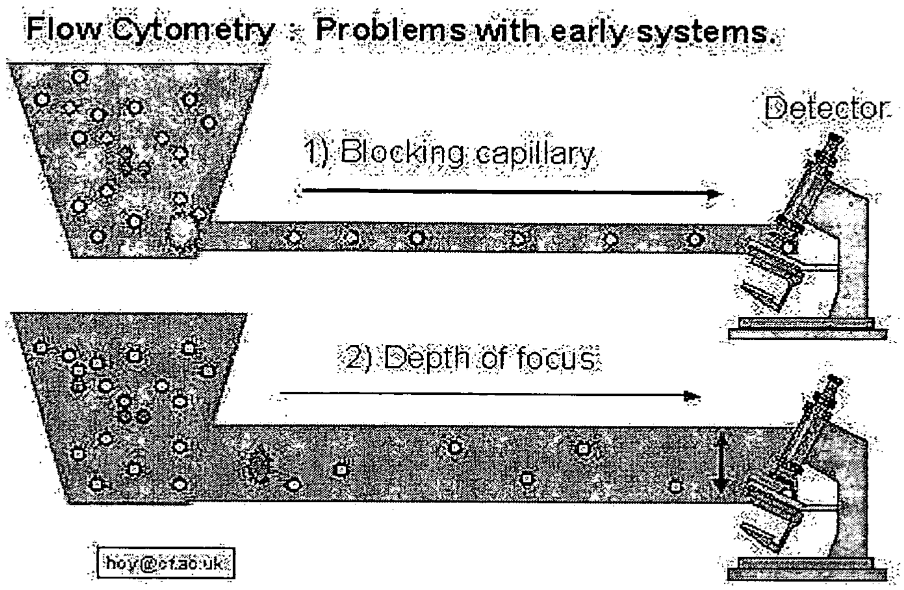
The appearance of flow cytometers in many routine clinical laboratories has resulted from the acceptance of flow cytometry in the field of immunophenotyping. They are now regarded by many as high speed automated microscopes rather than esoteric items of research equipment and as a consequence of this many traditional assays have been adapted for this technology. Several of these have been successful at exploiting the benefits of flow cytometry i.e. the rapid simultaneous measurement of several parameters on a cell by cell basis. This is in contrast to the more traditional 'biochemical' measurement of individual parameters to give a final mean value per cell. A major benefit of this approach has been to address the question of accurate enumeration of minor cell populations, which have always been problematic to estimate by manual microscopic methods.

This article aims to give a concise description of the principles of flow cytometry and sorting together with an indication of preparation required before specimens can be processed. The topics of data presentation, analysis and quality control is also covered because as the number of parameters handled by flow cytometers has increased an understanding of how the data can be visualized has become essential.

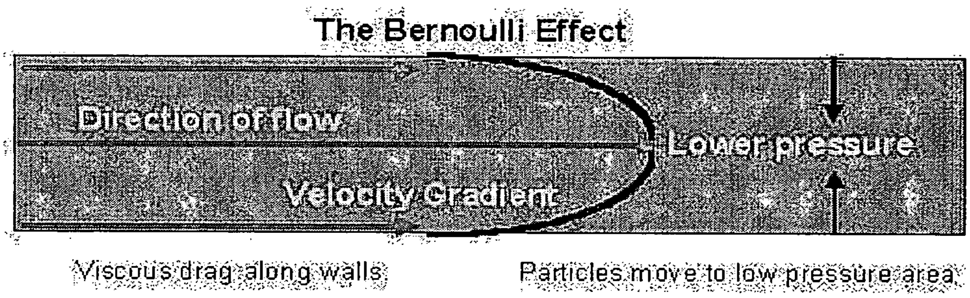
PRINCIPLES OF FLOW CYTOMETRY

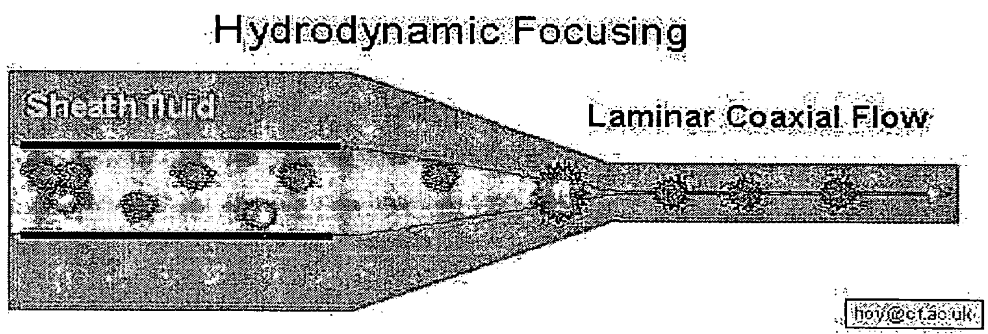
Fluidics and hydrodynamic focusing

Early attempts at flow cytometry consisted of apparatus designed to deliver cells (or particles) through a fine capillary tube with some optico-electric detector coupled to a microscope measuring their characteristics. This technique was plagued with the problem of large cells or clumps of cells blocking the capillary, attempts to use wide capillaries resulted in depth of focus effects at the point of examination.



A solution to this was found in combining the principles of laminar flow and hydrodynamic focusing.





Assuming laminar (non-turbulent) flow of liquid through a tube viscous drag at the boundary will result in a higher velocity nearer the centre, the velocity profile being that of a parabola. The Bernoulli effect associates such changes in velocity with inverse changes in pressure such that any particle in the fluid will move towards and remain in the centre. To prevent blocking, a suspension of cells can be introduced through a wide bore which is surrounded concentrically by a larger bore containing sheath fluid. By constricting this coaxial flow whilst maintaining laminar flow a focused stream of cells can be obtained as shown.

Physical constraints imply that to maintain focusing for particles of cellular dimensions the stream requires a velocity of several metres per second. At this speed a typical cell would traverse its own diameter in a few

microseconds which necessitates a rapid analytical system. This usually employs a highly focused laser beam for excitaion and sensitive photomultiplier tubes (PMTs) for detection.

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Excitation and collection optics

As stated in the introduction flow cytometers are essentially microscopes and they are configured to examine radiation at several wavelengths simultaneously. On some flow cytometers the laser beam interrogates the stream of cells in a flow cell constructed with optical windows as part of the coaxial arrangement, whereas on some cell sorters the stream is ejected though a nozzle before encountering the beam. Excitation is most common by an argon ion laser tuned to 488nm, which is close to the absorption maximum of the common fluorochrome fluorescein isothiocyanate (FITC). This wavelength is usually fixed for the small lasers installed on benchtop cytometers but on larger versions a range of discrete values is available from the ultra violet (351/363nm) to green (515nm) regions of the spectrum allowing a wider range of fluorochromes to be utilised. On more sophisticated cytometers two or more laser can be used to analyse the stream of cells in sequence enabling complex combinations of fluorochromes to be examined. Laser beams are usually focused to a spot between 10 and 60 microns in diameter and at typical flow rates cells will travers this in a few microsceconds. Experimental evidence suggests this is not detrimental to the cells viability or integrity.

Properties of Laser Light.
Coherent radiation at **discreet** wavelengths.

Some Common Sources.

Helium-Cadmium UV **325** nm

Argon Ion. UV **350/364** nm
Visible **458 465 472** 488 496 502 515 nm

Krypton Ion. UV **350/356** nm
Visible **468 476** 482 520 530 568 **647 676** nm

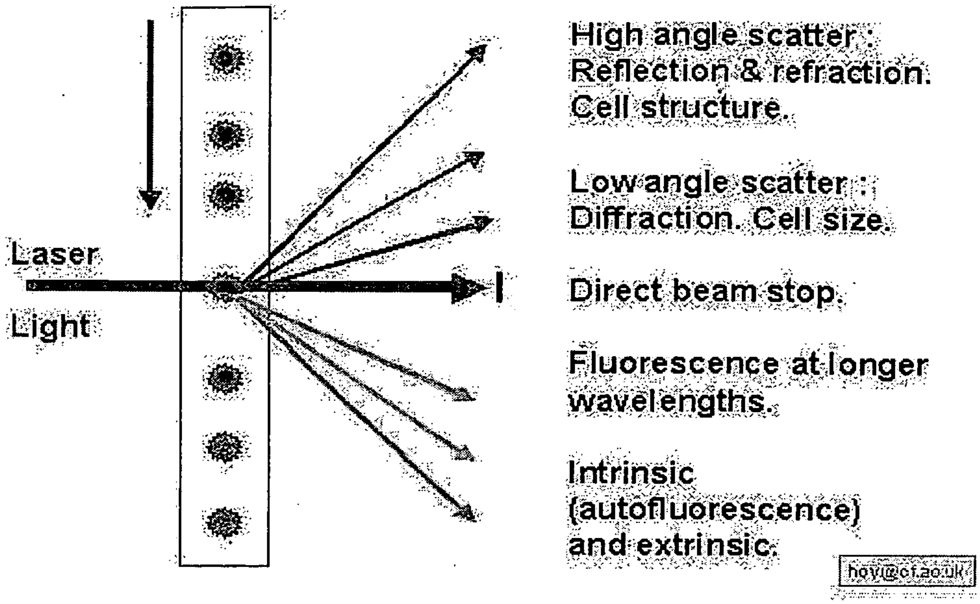
Helium/Neon 633 nm

Diode 635 nm

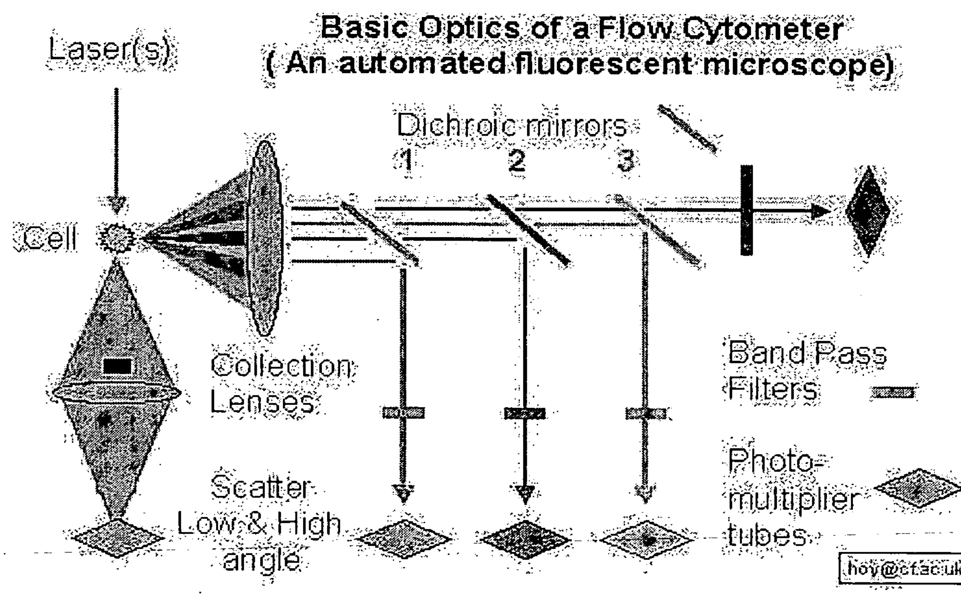
hoy@cf.ac.uk

As a cell passes through the laser beam several physical processes take place consisting of
1. Absorption, diffraction, refraction and reflection of the incident light and
2. Emission of longer wavelengths following excitation and relaxation of electronic states (fluorescence).

Diffraction is of significance close to the optical axis and is closely related to the size of the object passing through the laser beam.



After blocking the direct laser beam, any low angle scattered light is focused by a lens onto a photoelectric diode. This signal represents an important parameter commonly referred to as forward angle light scatter (FS or FALS). At higher angles refraction and reflection become increasingly important, these processes result from structural features of the cell such as granularity and surface convolutions. For convenience this measurement is made orthogonal to the stream and is referred to as side or 90° scatter (SS, SSC). Fluorescence measurements are also made in the orthogonal direction after separating various components by optical filtration. A typical arrangement for measuring four components is shown:



The orthogonal signal is processed by an arrangement of dichroic mirrors, these are normal interference filters

which when orientated at 45° to a signal reflect above (or below) a given wavelength and transmit below (or above) that wavelength. Before entering photomultiplier tubes (PMT's) signals are processed by wide bandpass filters selected to optimize the various fluorescent emissions an exception being that used for the side scatter signal which is usually a narrow bandpass filter (+/- 5nm) centered on the laser line being used. The magnitude of side scatter signals will be considerably greater than any fluorescence any is therefore easier to detect, for this an alternative to the dichroic mirror is a plane piece of optical glass which will reflect about 1% of the orthogonal signal into a PMT. The wide bandpass filters do not necessarily collect all the light from a particular fluorochrome, in general, if more than one fluorochrome is present their emission spectra will show some degree of overlap. The bandpass filters are therefore designed to minimise this overlap at the expense of loss sensitivity. Any residual overlap can eliminated electronically and will be discussed in the next section. On some instruments the filter system is fixed whereas others can be modified for different combinations of fluorochromes.

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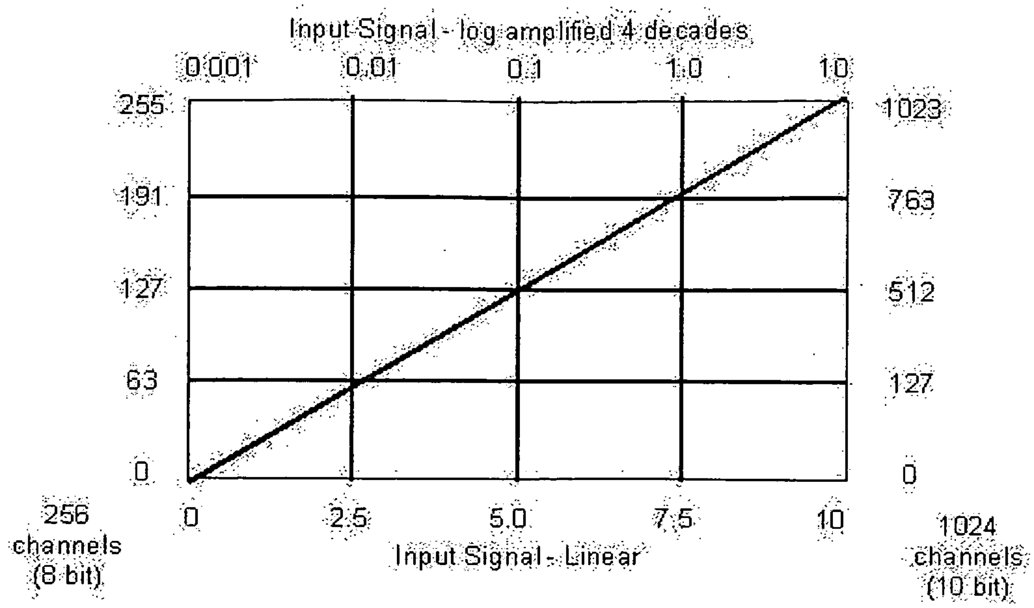
Signal Processing

This section deals with routine manipulations that can be applied to signals before they are saved for subsequent data analysis. The relationship between incident electrons and output voltage from PMT's is sigmoid i.e. the output voltage reaches a saturation level. The sensitivity is also a function of high voltage, if this is increased too far the dynamic range of a tube will be severely limited by the saturating level. After pre-amplification the final voltage is usually in the range 0-10.

The amplified signal can be used directly or subject to a logarithmic transformation which effectively increases resolution for low input level at the expense of high levels. The linear range of input voltage is transformed such that changes of equal magnitude represented by changes of equal difference. The number of orders of magnitude (or decades) over which the voltage is transformed is usually three or four, on some instruments it was possible to select five but this dynamic range is considered to be beyond the practical limits of the electronics employed. This is useful when attempting to measure fluorescent levels close to background and will be explained in more detail when considering data analysis.



The final stage before storing signals is converting from analogue to digital which effectively defines the resolution. For convenience many systems operate on an eight bit conversion giving 256 channels (usually numbered 0-255). This allows one measurement per byte (8 bits) and is sufficient resolution for most flow cytometric measurements. Ten bit conversion allows 1024 channels and is commonly used for DNA measurements to estimate ploidy and cell cycle parameters and also for investigating chromosomes where fine detail is essential.

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Having amplified and transformed signals from the various detectors a correction can be applied for any residual overlap of fluorescent spectra through the optical filters described in the preceding section. This process is referred to as compensation. If two fluorochromes, FITC and PE are being measured with spectral characteristics as shown: